Molecular Characterization of 1-Amino Cyclopropane 1-Carboxylate Deaminase Gene Produced By Drought and Salt Tolerant Bacterial Mutants and Their Plant Growth Promotion Activities on Legumes of Thar Desert, Rajasthan

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Introduction

Many environmental stresses including drought and salt stress limits the growth and productivity of crops particularly in arid and semi-arid areas. Rajasthan the largest state of India constitutes 60% of its area in Thar Desert. Its vegetation includes floral diversity, with 911 wild species belonging to 780 genera and 154 families. Geographically, Rajasthan lies between 23° 3' to 30° 12' longitude and 69° 30' to 78° 17' latitude. It occupies 342,239 km^2 land area, which is 10.41% of the total land area of India. The Thar Desert lies between 24° to 28° N latitude and 68° to 71° E longitude, occupying an area of about 200,000 km^2. Ecosystem of Rajasthan's arid and semi-arid regions is mainly affected by high temperature, soil salinity, drought, low pH and metal toxicity. Legumes (*Vigna aconitifolia*, *V. radiata*, *Cicer arietinum*, *Glycine max* L. Merrill etc.) grown in arid and semi-arid regions of Rajasthan faces problem of abiotic stresses like salinity, alkalinity, high temperature and draught (http://dst.rajasthan.gov.in/). The effects of these environmental stresses can be quantified as reduction in plant growth and concomitant reduced dry weight, stomatal conductance decreases, thus reducing water loss from leaves and photosynthetic rate is reduced due to decreased intercellular CO_2 concentration (Mahajan and Tuteja, 2005).

Drought and salinity stress show a high degree of resemblance in terms of physiological, biochemical, molecular and genetic effects (Sairam and Tyagi 2004). Physiological drought occurs when sodic salts increase in the soil solution are high enough to limit water uptake from plant roots due to low water potential, thereby inducing drought stress (Carrow and Duncan 1998). Both stresses lead to cellular dehydration, which causes ionic imbalance, osmotic stress and removal of high water content from the cytoplasm into the intracellular space resulting in a reduction of the cytosolic and vacuolar volumes (Nakashima et al. 2014). Early responses to water and salt stress are largely identical except for the ionic component in the cells of plants under salt stress. These similarities include metabolic processes such as, decrease in photosynthesis rate and increase in levels of plant hormonal processes (e.g., abscisic acid (ABA), indole-3-acetic acid (IAA), Ethylene etc.).

Drought and salinity stress extensively associated with elevated release of endogenous ethylene in the higher plants which responsible for growth inhibition (Mayak et al., 2004a; Arshad et al., 2008; Li et al. 2013). It is a simple gaseous plant

hormone that is involved in the regulation of many physiological responses, includes root and shoot growth, seed germination, flower development, ripening of fruits and senescence of plant organs (Arshad and Frankenberger 2002) and it was also regarded as a "stress hormone" because ethylene synthesis in plants is increased under a number of biotic and abiotic stresses (Li et al. 2013).

Ethylene level enhanced during salt and drought stress which is unfavorable for plant growth, and may be alleviated by using beneficial plant growth promoting bacteria (PGPB). Glick et al., 2007, elaborately emphasized role of 1-amino cyclopropane 1-carboxylate (ACC) deaminase gene producing microbial strain that alleviate ethylene stress whereby plant survive under salt stress. PGPB are a poorly investigated/unexplored group of microorganisms in arid and semi-arid zones of Rajasthan that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial areas. The mechanisms through which PGPB exist and respond to their surroundings must be better understood in order to be more predictive about which higher plants to seek, study, and spend time isolating micro floral components. Exploitation of PGPB-plant interactions can result in the promotion of plant health and can play a significant role in low-input sustainable agriculture applications for both food and non-food crops. PGPB are naturally occurring soil bacteria that colonize with plant roots and benefit plants by providing growth promotion. Inoculation of plants with certain strains of PGPB at an early stage of development improves biomass production through direct effects on root and shoots growth such as enhancement of seedling germination, stand health, plant height, and shoot weight, nutrient content of shoot tissues, chlorophyll content, and increased nodulation in legumes.PGPB that grow in presence of high salt and drought conditions may alleviate such stress. PGPB also enables plants to survive in stress conditions by the mechanism known as Induced Systemic Tolerance (IST). IST is an important process to overcome the harmful effects of abiotic stress. It is a PGPB induced physical and chemical changes in plants that result in enhanced tolerance to abiotic stress viz; draught, salt, heavy metals, cold, heat shock and fertility stress. PGPB reduces the detrimental effects of stress by secreting stress releasing molecules. IST results from the production of plant growth promoting hormones like cytokinin, antioxidants like catalase, ACC deaminase activity or volatile organic compounds (Yang *et al.*, 2009).

Development of overproducing PGPB mutants or strain improvement could be a more beneficial way to increase its chances of survival in stress. Mutant is an individual, organism, or new genetic character, arising from an instance of mutation, which is a base-pair sequence change within the DNA of a gene or chromosome of an organism resulting in the creation of a new character or trait not found in the wild type. Mutations allow new versions of DNA combinations to occur. There are various mutagens present such as Mitomycin C (MMC), ethyl methane sulfonate (EMS), diethylsulfate (DES), in which a potent chemical mutagen available is N-methyl-N′-nitro-N-nitrosoguanidine (NTG) which induces the high frequency of mutation at DNA level. NTG creates the changes in nucleic acid sequences by mutations include substitution of nucleotide base-pairs and insertions and deletions of one or more nucleotides in DNA sequences (Mishra and Goel,1999).

Hypothesis and objectives of the present research

Improvement of our understanding of plant-microbe interaction under a variety of abiotic stresses is essential because several studies have been carried out on inoculation of beneficial microbes under normal conditions but the specifics of these interactions, specifically under varying adverse environmental conditions are often lacking. The studies presented in this thesis were designed to investigate the induced systemic tolerance in legumes especially, *Vigna radiata* and *Glycine max* through ACC-deaminase containing drought and salt tolerant wild and mutated bacterial strains. So, keeping this in view, a series of experiments has been conducted under control conditions, to achieve the following:

The specific objectives of the studies reported in this thesis are as follow:

- Assessment of salt and osmotic stress tolerance and characterization of plant growth-promotion traits of stress tolerant bacterial strains.
- Molecular characterization of 1-amino cyclopropane 1-carboxylate deaminase and other plant growth promoting bacterial genes
- Bacterial strain improvement for different plant growth promoting traits by mutagenesis
- To evaluate the potential of *P-simiae* mutated strain AU-M4 to document their affectivity for enhancing tolerance against abiotic stresses in leguminous plants.
- Expression pattern of differentially expressed drought and salt stress responsive genes in soybean and mungbean plants

Review of literature

Plant growth promoting bacteria are defined as free-living soil, rhizosphere, rhizoplane and phylosphere bacteria that, under some conditions, are beneficial for plants. Plant associated bacteria can contribute to the health, growth and development of plants. Examples of bacteria which have been found to enhance plant growth include *Pseudomonasspp.*, *Enterobacter*, *Bacillus spp.*, *Erwinia, Azospirillum and Arthrobacter*.The use of beneficial microbes in agricultural production systems started long time ago, and there is increasing evidence that beneficial microbes can enhance plants' tolerance to these environmental stresses, which include salt stress (Egamberdieva *et al.*, 2008), drought stress (Zahir *et al.*, 2008), nutrient deficiency, and heavy metal contaminations (Shenge*t al.,* 2005). Based on different plant growth-promoting properties, viz., indole-3-acetic acid production (IAA), inorganic P-solubilization and 1-aminocyclopropane1-caboxylic acid (ACC) we may employ beneficial microbes for cultivation of plants under stress. Choudhary *et al.* (2011) elaborately described role of microbes in alleviation of stress in form of biotechnological perspectives together with mechanistic approaches. Barassi *et al.* (2006) emphasized removal of abiotic stress (salt) alleviation by employing plant growth-promoting bacteria (PGPB). Under stress, IAA is responsible for an increase in root length with surface area, is responsible for enhanced uptake of available nutrients (Egamberdieva and Kucharova 2009).

The gaseous plant hormone ethylene (C_2H_4) synthesized from the precursor 1-aminocyclopropane- 1-carboxylic acid (ACC) is involved in developmental processes in plants, such as tissue differentiation, lateral bud development, seedling emergence, flower senescence, root hair development and elongation, fruit ripening, production of volatile compounds responsible for aroma in fruits (Abeles *et al.*, 1992; Frankenberger and Arshad 1995; Spaink 1997; Bleecker and Kende 2000). Under normal conditions, plants produce required levels of ethylene, conferring beneficial effects on plant growth and development; however, in response to biotic and abiotic stresses there is often a significant increase in the endogenous ethylene production that has adverse effects on plant growth and is thought to be responsible for senescence in plants (Nayani *et al.*, 1998; Ali *et al.*, 2012). PGPB strains possess the enzyme ACC-deaminase and this enzyme can cleave the plant ethylene precursor ACC to ammonia and α-ketobutyrate

thereby lowers the level of ethylene under various biotic and abiotic stresses (Glick et al. 1998) such as salt stress (Cheng *et al.*, 2007, Mayak *et al.*, 2004a; Zahir *et al.*, 2009), drought stress (Mayak *et al.*, 2004b), heavy metal stress (Belimov *et al.*, 2005; Stearns *et al.*, 2005). ACC deaminase-containing plant growth promoting rhizobacteria lowers the level of ACC in the stressed plants, thereby limiting the amount of stress ethylene synthesis.

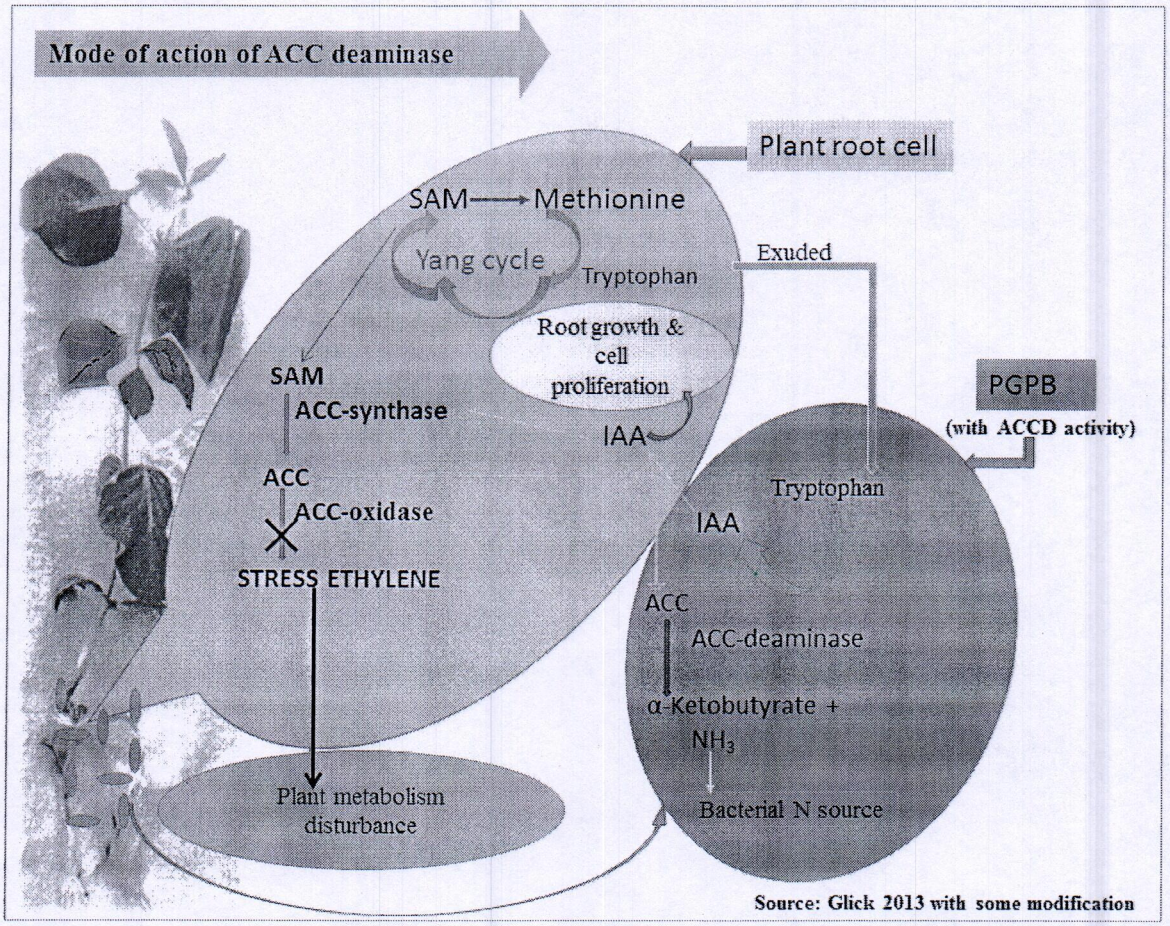

Fig. A model describing the role of bacterial ACC deaminase in the promotion of plant growth, by lowering potentially deleterious ethylene levels in the plant Glick et al. (1998, 2007).Where, ACC = 1-aminocyclopropane-1-carboxylate; IAA = Indoleacetic acid; SAM = S-adenosyle-methionine

Beneficial bacterial mediated growth promotion in plants can successfully be obtained by direct growth enhancement or alleviating the environmental conditions (Glick *et al.*, 2007). PGPB moreover make the plant to grow in nitrogen deficient soil by fixing nitrogen (Dobbelaere *et al.*, 2003). These may also indirectly promote growth by production of antimicrobial compounds to decline or prevent the harmful effects of

nonpathogenic, deleterious microorganisms (Raaijmaker *et al.*, 2002) and siderophore production to scavenge iron and thereby reducing iron deficiency (Rajkumar et al. 2010).These pseudomonad strains have excellent root colonizing ability, andproduce a wide range of enzymes and metabolites that favour the plant withstand under varied biotic and abiotic stress conditions (Choudhary et al. 2012). The plant-associated habitat is a dynamic environment in which many factors may affect the structure and species composition of the bacterial communities that colonize plant tissues. Some of these factors are seasonal changes, plant tissue, plant species and cultivar, soil type and interaction with other beneficial or pathogenic microorganisms. An understanding of the structure and species composition of plant-associated bacterial populations is fundamental to understanding how plant-associated biological processes are influenced by environmental factors and, consequently, has important biotechnological implications. Plant growth and development cannot be adequately described without acknowledge microbial interactions.

Strain improvement through mutational technique for development of overproducing PGP traits is most effective strategy under stressed environment to increase plant growth and productivity (Latt et al. 2013; Ali et al. 2014). Many researchers have also developed mutants of *Pseudomonas* spp. for enchantment of ACC-D activity and exopolysaccharide (EPS) productions, which were found to promote better plant growth under adverse conditions as compared to its wild strain (Mishra and Goel 1999; Ali et al. 2014; Kasotia and Choudhary 2014; Kumari et al. 2016). In addition, Trivedi and Sa (2008) developed the mutants of *P. corrugate* (NRRL B-30409) with increased Pi-solubilization activity and plant growth under lower temperature stress. Similarly, Mohamed and Ebaid (2011) developed PGPR mutant of *Bacillus polymyxa* for production of high Pi-solubilization and Fe uptake as compared to the wild type strain, which increased plant root and shoot dry weight, P-uptake and Fe-uptake in shoot.

Adopted methodology

1. **Maintenance of bacterial strains**

   Recovered isolates will be maintained on nutrient agar plates amended and plates will be incubated in an incubator at 28 ± 2°C for 24 to 36 hours. A 2.5 ml, autoclaved mixture of glycerol + nutrient broth (1:1 v/v) was added to 5 ml of overnight grown culture in the same medium. One ml of this mixture will be suspended in cryovial and preserved at – 20°C.

2. **Screening of salt and drought tolerant bacteria**

   The bacterial strains will be grown on nutrient broth under varying polyethylene glycol (PEG) concentration ranging from (0%, 5%, 10% and 15%) and NaCl salt concentration (0mM, 200mM, 400mM and 600mM) for 72 hrs. at 28°C. Isolates, which will be able to tolerant high level of drought and salt stress, will be used for the further study.

3. **ACC Deaminase activity and other plant growth promotion traits**

   Screening for ACC deaminase activity of drought and salt tolerant bacterial isolates will be determined by measuring the amount of α-ketobutyrate produced by the cleavage of ACC by ACC deaminase (Penrose and Glick 2003) with some modification. Isolates, which will be positive for ACC deaminase will screened for other plant growth promoting traits such as IAA production (Patten and Glick, 2002), phosphate solubilization (Gaur, 1990) and exopolysaccharide production (De Vuyst *et al.*,1998).

4. **Molecular characterization ACC-deaminase and other PGP bacterial genes**

   For molecular identification, the selected salt and drought tolerant bacterial strains were subjected to partial or full sequencing of the 16S ribosomal RNA (16S rRNA) gene. For PCR amplification of 16S rRNA gene of bacterial isolates, DNA purified, prepared for sequencing and after getting sequencing results, bacterial strains were identified by using software "ncbi gene blast". After identification of bacterial strain, different bacterial genes such as *acdS, trp, gad, sid and p5cr* were amplified by PCR using corresponding gene primers.

5. **Strain improvement through random mutagenesis**

   Bacterial cells will be treated with different chemical mutagens such as NTG and EMS etc. with different concentrations under both type of stress according to Mishra and Goel (1999) and Mohamed and Ibrahim (2011) for high PGPR activities viz; ACC deaminase activity, phosphate solubilization, and IAA production.

6. **Screening of positive mutants for enhanced PGP traits**

   After various mutagenic treatments, all the select isolates will be tested for over-production of three PGP traits (ACC-deaminase activity, IAA production and Pi-solubilization) compared to wild type strain in the presence of NaCl concentration and PEG concentration.

7. **Bacterial-elicited induced systemic tolerance against salinity and drought stresses in experimental plants (soybean and mungbean)**

   Selected strains will be analyzed for plant growth promoting activities in experimental plants that are normally challenged to stresses. Both wild and mutant bacterial inoculum will be prepared by harvesting bacterial cells from 24h grown cultures on nutrient plates at 28°C. The inoculum will then be suspended in sterile physiological saline and inoculated in soil in controlled conditions and checked for their potency to promote plant growth under drought and salt stress conditions.

FINDINGS

Soybean (*Glycine max* L.) and mungbean (*Vigna radiata* L.) both are important conventional food legume crops in Rajasthan. Among several factors which contribute to low productivity of both leguminous plants, elevated levels of ethylene in plant could be one of the major growth limiting factors under severe salinity and drought. It is established fact that accelerated levels of ethylene are produced in plant in response to biotic and abiotic stresses, which inhibit root growth and shoot growth. Some PGPB are capable of lowering the stress ethylene levels through their ACC-deaminase activity and thus enhance root growth and, consequently shoot growth and yield. It is very likely that the use of PGPB containing ACC-deaminase could be effective for promoting growth, and yield of soybean and mungbean. However, the PGPB strain with multiple PGP trains traits could be more useful under diverse conditions compared to a strain containing single trait. In the present study, PGPB *P. simiae* AU containing ACC-deaminase and their respective ACC-deaminase overproducing mutated strain AU-M4 were applied for their potential role to enhanced the growth of soybean and mungbean by conducting experiments under axenic (controlled) and stressed (0.4 M NaCl and 10% PEG) conditions.

A series of experiments was conducted to evaluate the potential of PGPB containing ACC-deaminase for improving the soybean and mungbean under salt and drought-affected conditions. These experiments and their results are summarized below:

1. A number of nine bacterial strains (CBS-1, CSS-1, CCS-2, CHS-1, CJS-2 and CKS-2, AU, AK-1 and SJ-5) were selected for this study; isolated from rhizospheric soil of soybean and other plants.
2. Assessment of salt and drought tolerance of all 9 strains was conducting: The all bacterial strains were grown at varying NaCl salinity levels ranging from 0mM, 100mm, 200mM, 400mM and 600mM and at varying PEG drought levels ranging from 0%, 5%, 10% and 15% and the optical density was measured. The results showed that the out of 9 strains three bacterial strains namely AU, AK-1 and SJ-5 were able to show growth at each NaCl and PEG stress levels, however growth varied in their ability to tolerate the NaCl and PEG stressed conditions.
3. The bacterial strains AU, AK-1 and SJ-5 were further screened on the basis of different PGP activities such as ACC metabolism, and other PGP activities including IAA production, Pi-solubilization and EPS production assay.

Results of the bioassay showed that all the bacterial strains had the ability to utilize ACC as a sole source of nitrogen but with variable degree of effectiveness.

4. The strains *P. simiae* strain AU, which showed efficient ACC-deaminase activity, IAA production and Pi-solubilization activity was further treated with NTG, EMS and UV-NTG mutagens and selected to study the effect of mutation on their all three PGP activities and its positive role in plants stress alleviation.
5. After subjecting *P. simiae* strain AU to different mutagenesis with NTG, EMS and UV-NTG all selected mutated cells were characterized for different PGP activities, viz. ACC-deaminase, IAA production and Pi-solubilization accordingly in the presence of 10% PEG and 0.4M NaCl concentration wit compared to wild strain AU in same conditions (Fig 4.5). For ACC-deaminase detection and IAA production out of 25 selected mutated cells one mutant colony AU-M4 was recorded highest ACC-D activity.
6. Based on biochemical estimation of PGP activities and aim of my research work only one mutant AU-M4 was found positive for produce ACC-deaminase production and other two IAA and Pi-solubilization PGP activities more than wild type strain AU. Therefore, we selected only one mutant AU-M4 and its wild strain *P. simiae* strain AU for further plant growth experiments for their potential role to alleviate salinity and drought stresses and enhances the growth of selected plants (soybean and mungbean) through various biochemical and molecular mechanisms including: improve growth parameters, antioxidant productions, phytohormone production and differential expression of different stress responsive genes.
7. The overall study has established that mutagenesis through NTG is a good method for improving the PGP features of the *P. simiae* strain AU and secondly mutated bacterial cells inoculated soybean and mungbean plantlets are show more induced systemic salinity and drought tolerance mechanism. It seems that mutated strain AU-M4 enhance osmotic adjustment by the accumulation of osmolytes which could contribute to maintaining water potential encouraging to the water movement from soil into the roots. In addition, inhibition of stress ethylene level in bacterial inoculated plant tissue relieves detrimental effects of salinity and drought stresses in soybean and mungbean plantlets. Hence, the development of overproducing mutant of *P. simiae* strain AU through NTG mutagenesis alleviated the salinity and drought stresses and ameliorates soybean and mungbean plant growth.

Significance of work

- Agriculture production systems are liable to changing environmental conditions due to global warming; root-colonizing bacteria have been shown increase tolerance to abiotic stresses in host plants as well as able to enhanced resistance to biotic stress.
- Use of microbial inoculants is viewed as a more appropriate technology in many of the less-developed countries of the world where agricultural chemicals are relatively expensive and labor is inexpensive.
- PGPB will only gradually and modestly displace chemical solutions to agricultural and environmental problems.
- Mutants having enhanced PGP activities are developed which may help the plant to survive in drought and salt stressed conditions.
- These bacterial strains elicited induced systemic tolerance (IST) to physical and chemical changes in the plants that enhanced tolerance to abiotic stress.

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